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FMRFamide and GABA Produce Functionally Opposite Effects on Prey-Capture Reactions in the Pteropod Mollusk *Clione limacina*

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Abstract. The effects of FMRFamide and gamma-aminobutyric acid (GABA) on prey-capture reactions in *Clione* and on cerebral **A** and **B** neurons, which control opposite movements of prey capture appendages, have been studied. FMRFamide hyperpolarized **A** neurons and depolarized and increased spike activity in **B** neurons. FMRFamide thus had a reciprocal effect on **A** and **B** neurons, triggering buccal cone withdrawal. In addition, FMRFamide inhibited swimming, acceleration of which is a component of feeding arousal. Many neurons throughout the central nervous system showed FMRFamide immunoreactivity. Dense networks of immunoreactive fibers were localized in the head wall, buccal mass and in buccal cones, adjacent to striated longitudinal muscle cells. In wings, immunoreactive processes were found mainly in association with smooth retractor muscles. GABA depolarized and activated **A** neurons but hyperpolarized and inhibited **B** neurons. The overall effect of GABA thus resulted in extrusion of buccal cones. Both direct GABA responses and inhibitory postsynaptic potentials (IPSPs) induced in **B** neurons by **A** neuron activity were chloride-mediated. However, picrotoxin and bicuculline did not block IPSPs or direct GABA responses in **B** cells.

Introduction

The pteropod mollusk *Clione limacina* is a highly specialized carnivore that feeds on shelled pteropod mollusks of the genus *Limacina* (Wagner, 1885; Lalli and Gilmer, 1989). To catch the prey, *Clione* uses three pairs of oral appendages, called buccal cones, that are normally cone shaped and covered by skin folds. Contact with the prey induces very rapid eversion of the buccal cones, which then become tentacle-like and seize the shell of the prey. The eversion and elongation of buccal cones is primarily due to hydraulic inflation. After capturing its prey, *Clione* uses its buccal cones to position the *Limacina* shell aperture over its mouth and uses two clusters of specialized chitinous hooks and the radula, to extricate the *Limacina* from its shell, swallowing it whole. Feeding behavior of *Clione* also involves a significant increase of locomotory activity: swimming changes from slow to fast (Litvinova and Orlovsky, 1985; Hermans and Satterlie, 1992).

Two groups of motoneurons in the cerebral ganglia of *Clione* have been identified as directly involved in the control of prey-capture reactions (Norekian and Satterlie, 1991a, b, 1993). The first group consists of electrically coupled cells, called **A** neurons, which form an integral network that is silent in nonfeeding animals. Upon activation, **A** neurons evoke opening of the oral skin folds and extrusion of the buccal cones. The second group of motoneurons (**B** neurons) evokes retraction of buccal cones into the head. In nonfeeding animals, **B** neurons show regular spike activity that maintains the buccal cones in the withdrawn position. **A** and **B** neurons thus serve antagonistic functions. When active, **A** neurons induce a powerful inhibitory input to **B** cells, which terminates **B** cell firing. This reciprocal arrangement of firing activities directly determines the behavioral state of the buccal cones. Any changes in the activities of **A** and **B** neuron groups produce significant changes in the behavioral output and are thus very important for realization of feeding behavior.

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A and B neuron activities and behavioral responses of buccal cones can be controlled or modulated by different neurotransmitters. The roles of various neurotransmitters in realization of specific behaviors have been investigated in a variety of invertebrates. Serotonin, for example, appears to act as a central transmitter-modulator for feeding arousal and activation of feeding behavior (*e.g.*, Lent *et al.*, 1989; Kupfermann and Weiss, 1981). A behavioral role has also been demonstrated for several other neuroactive substances, including the neuropeptide FMRF-amide (Murphy *et al.*, 1985; Bulloch *et al.*, 1988; Cooke *et al.*, 1985; Cooke and Gelperin, 1985).

The primary goal of this study is to describe the effects of the neuropeptide FMRFamide and gamma-aminobutyric acid (GABA) on the system of A and B neurons underlying prey-capture reactions in *Clione*, and on its behavioral output. FMRFamide-containing cells and fibers in the central nervous system (CNS) and peripheral tissues of *Clione* are localized immunohistochemically. These data allow discussion of the role of FMRFamide and GABA in feeding behavior in *Clione*. Preliminary results of some GABA effects have been published (Norekian and Satterlie, 1991a, b).

Materials and Methods

Behavioral and physiological experiments were carried out at Friday Harbor Laboratories, University of Washington, in the spring and summer. Adult specimens of *Clione limacina*, 1–3 cm in body length, were collected from the breakwater and held in large beakers of seawater at 10–13°C. Morphological work was carried out at Arizona State University.

Preparation

Electrophysiological experiments were performed on preparations consisting of the head, central nervous system, and wings. All nerves running from the central ganglia to the head and to the wings were intact, while body nerves were cut. The preparations were tightly pinned to a Sylgard-coated petri dish with cactus spines (*Opuntia sp.*). Prior to recording, ganglia were partially desheathed by bathing the preparation in a 1 mg/ml solution of protease (Sigma type XIV) for approximately 5 min, followed by a 30–60 min wash. The solution in the recording chamber was changed with the aid of a peristaltic pump that allowed constant perfusion of solutions at a rate of 1 ml/min (chamber volume = 5 ml). Solutions were prepared using filtered natural seawater.

Electrophysiological recordings

For intracellular recordings, glass microelectrodes were filled with 2 *M* potassium acetate, and had resistances of 10–20 *M*Ω. Electrophysiological signals were amplified, displayed, and recorded using conventional techniques. Intracellular stimulation was provided via amplifier bridge circuits. Electrodes were filled with 3 *M* potassium chloride for the experiments with high intracellular chloride; chloride leakage from the electrodes caused the required increase in intracellular chloride concentration.

Transmitter application

To understand if a recorded neuron was directly sensitive to a neurotransmitter, three methods were used. The first involved chemical isolation of neurons by bathing the preparation in high concentrations of divalent cations. Solutions used included 333 m*M* magnesium chloride, 2 m*M* cobalt chloride, and 0.5 m*M* cadmium chloride. Magnesium chloride solution blocks chemical synaptic connections in the *Clione* preparation as demonstrated in the swimming system (Satterlie, 1989).

The second method included mechanical isolation of identified neurons. In these experiments, the ganglionic sheath was detached from the surface of neurons by exposure to 2 mg/ml protease for 10–15 min. The sheath was then mechanically removed and the preparation washed for 30–60 min. The appropriate neuron was penetrated with a recording microelectrode and slowly pulled from the ganglion. Axons that might still have provided synaptic connections with the CNS were then cut.

The third method involved local application of transmitter directly onto the cell body of the recorded neuron. Microiontophoresis was used for GABA, and diffusion microapplication was used for both FMRFamide and GABA. For microiontophoresis, glass micropipette electrodes with tip diameters of 1 μm were filled with a 1 m*M* solution of GABA prepared in distilled water. The amplitude of negative current pulses used to eject GABA was 10 to 100 nA with durations of 100 to 1000 ms. For diffusion microapplication, glass pipettes with tip diameters of 2–3 μm were filled with a 1 m*M* solution of the transmitter prepared in seawater and the electrode tip placed close to the cell surface. Transmitter was released from the tip of the electrode by diffusion. When the electrode tip was moved away from the recorded neuron, the effects were eliminated. Addition of neurotransmitters and antagonists to the bathing solution was accomplished with a graduated 1 ml pipette. The final concentrations were calculated from the known volume of injected solution and the known volume of the recording dish. For use of GABA antagonists, the preparations were bathed in high Mg^{++} saline containing the required concentration of antagonist for 5–15 min. Picrotoxin, bicuculline, bicuculline methbromide, bicuculline methchloride, and pentylenetetrozole were purchased from Sigma Chemical Company or Research Biochemicals Inc. Picrotoxin, bicuculline

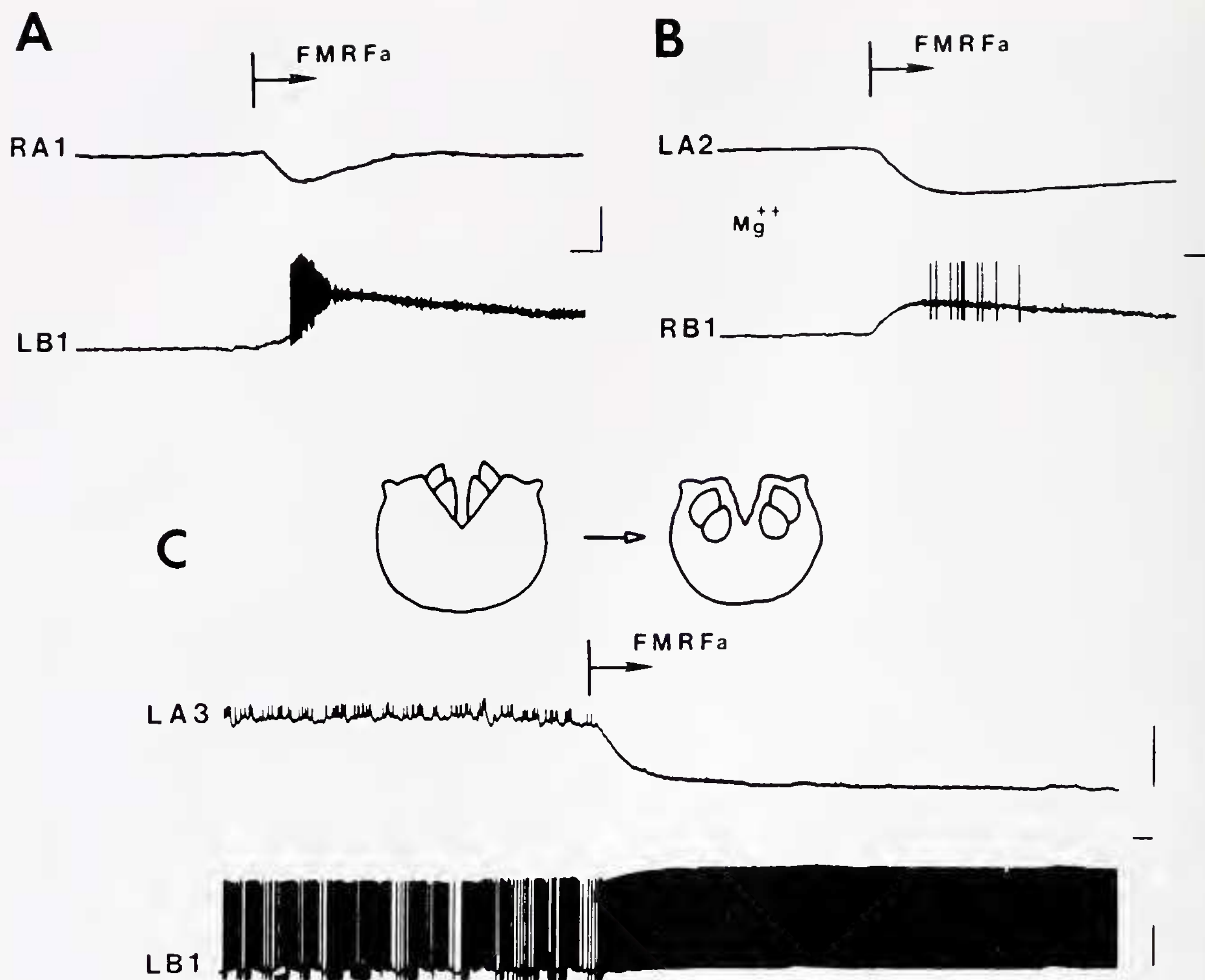

Figure 1. Effects of FMRFamide on A and B neurons. (A) Bath-applied 8 μM FMRFamide produced hyperpolarization of an isolated **RA1** neuron and depolarization of an isolated **LB1** neuron. The peptide was applied in constantly perfused seawater. Scale bars = 15 mV, 3 s. (B) In high Mg^{++} seawater, which terminated spontaneous firing of neuron **RB1**, application of 10 μM FMRFamide also hyperpolarized neuron **LA2** and depolarized and activated neuron **RB1**. Scale bars = 15 mV, 2 s. (C) Application of 10 μM FMRFamide in normal seawater resulted in hyperpolarization of neuron **LA3** and a significant increase of spike activity in neuron **LB1**. The line drawings schematically show the head in dissected preparations before and after FMRFamide application. Before application the buccal cones were slightly relaxed; after application they were completely withdrawn. Scale bars = 15 mV, 2 s.

methbromide, bicuculline methchloride, and pentylenetetrozole were diluted directly in seawater, while bicuculline first was diluted in 0.25 ml of alcohol and then in seawater. All GABA antagonist solutions were prepared immediately before use. In these experiments, GABA was prepared in a solution of the antagonist and applied with the 1-ml pipette. In this way, the final concentration of antagonist was not changed when the GABA was added. The same procedure was performed during tetraethylammonium or 4-aminopyridine (Sigma) and FMRFamide application.

Immunocytochemistry

Dissected preparations were fixed overnight in 4% paraformaldehyde in phosphate buffer, washed for 12 h in phosphate buffered saline (PBS), and pre-incubated in PBS containing 0.01% Triton X-100 and a 5% solution of goat serum in PBS/Triton X-100. Tissue was incubated in a 1:500 dilution of anti-FMRFamide antibody (INCSTAR) in goat serum/PBS/Triton for 48 h. Following a series of PBS washes, tissue pieces were incubated in a 1:20 dilution of fluorescein-labeled goat anti-rabbit secondary antibody (Kirkegaard & Perry) for 12 h. Another series

of PBS washes was followed by infiltration of a mounting medium consisting of 1 part 50 m*M* Tris buffer (pH 9.5) and 9 parts glycerol. Tissue pieces were viewed and photographed in whole mount in a Nikon fluorescence microscope with epifluorescence and standard FITC filters.

For electron microscopy the tissue was fixed in 2% glutaraldehyde in phosphate buffer instead of 4% paraformaldehyde. A similar procedure was used for applying the primary antibody. After washing, a peroxidase-labeled avidin-biotin secondary antibody system was used (Vectastain ABC elite kit). The peroxidase was reacted with diaminobenzidine, and the tissue was washed and fixed in 1% osmium tetroxide in phosphate buffer for 1 h. The tissue was washed, dehydrated in an ethanol series and propylene oxide, and embedded in Epon. Thin sections were cut on a Porter-Blum MT-2B microtome and examined in a Philips EM201 electron microscope. To test for the specificity of the FMRFamide immunostaining, two different controls were used. First, primary antibody was omitted from the procedure. Second, primary antibody was incubated overnight with 10 μM FMRFamide before use. In both cases, no labeling was detected.

The total number of preparations used in this study was 90.

Results

Effects of FMRFamide

FMRFamide, at 1–2 μM and above, hyperpolarized the cerebral **A** neurons and depolarized the **B** neurons (Fig. 1). To assess whether **A** and **B** neurons directly responded to FMRFamide, the peptide was tested on isolated somata of identified **A** and **B** neurons. Isolated **A** neurons demonstrated hyperpolarizing responses to application of FMRFamide, but isolated **B** neurons showed depolarizing responses (Fig. 1A). Similar responses were observed in high Mg^{++} solution (Fig. 1B).

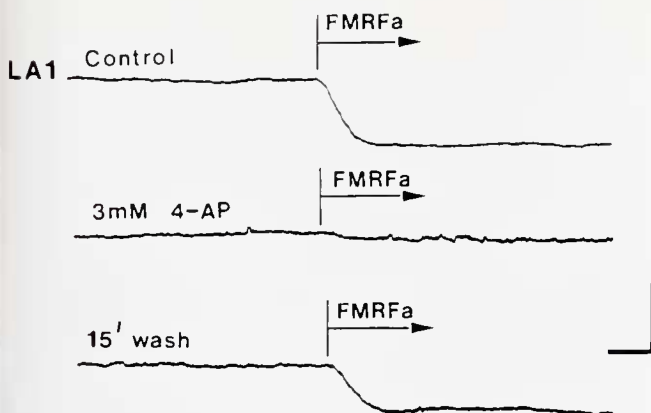

Figure 2. Blocking of FMRFamide hyperpolarizing responses in **A** neurons by 4-aminopyridine. Effects of 50 μM FMRFamide on neuron LA1 before, 1 min after 3 m*M* of 4-AP was applied externally, and after 15 min of washing in seawater. The entire experiment was conducted in high Mg^{++} seawater. Scale bars = 5 mV, 2 s.

Figure 3. Fast and slow inhibitory postsynaptic potentials recorded in **A** neurons. Scale bars = 15 mV, 2 s.

Bath application of FMRFamide in seawater produced hyperpolarization of **A** neurons and a 5- to 10-fold increase of **B** neuron spike activity (Fig. 1C). FMRFamide thus had a reciprocal effect on the activities of **A** and **B** neurons, which was visually correlated with the buccal cones being held in a withdrawn position (Fig. 1C). In all experiments, strong withdrawal reactions of buccal cones were observed for the duration of FMRFamide application.

In addition to FMRFamide and GABA, we also tested the effects of serotonin, dopamine, acetylcholine, SCP_B, and L-glutamate on the cerebral **A** and **B** neurons. FMRFamide was the only agent that hyperpolarized the **A** neurons; all the others produced depolarization. **A** neuron hyperpolarization, evoked by bath application of 5–10 μM FMRFamide, was 10–15 mV from the normal resting potential of −60 to −65 mV. Tetraethylammonium (TEA) and 4-aminopyridine (4-AP), known to block different voltage-sensitive K^+ currents (see Hille, 1992), antagonized the FMRFamide-evoked hyperpolarization (Fig. 2). Hyperpolarization produced by 50 μM FMRFamide was completely blocked by external perfusion of 10 m*M* TEA or 3 m*M* 4-AP after 2 min ($n = 9$). This effect was reversed by washing for 15–20 min in seawater.

Two types of inhibitory postsynaptic potentials (IPSPs) were recorded in **A** neurons: fast and slow (Fig. 3). Fast IPSPs had durations of less then 1 s, and amplitudes up to 5 mV; they appeared with frequencies of up to 3 Hz. Slow IPSPs had durations between 20 s and 1 min and amplitudes up to 15 mV. The source of neither type of IPSPs is known, but FMRFamidergic neurons may be involved.

One important component of natural feeding arousal in intact *Clione* is acceleration of swimming. For this reason the effect of FMRFamide on the swimming system was tested. Perfusion of 5 μM FMRFamide or higher concentrations completely blocked swimming activity, as noted through behavioral observation of wing movements and by recording electrical activity of swim motoneurons

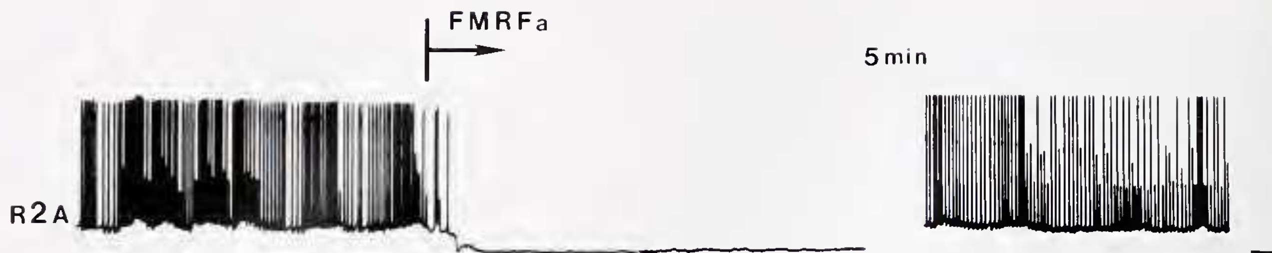

Figure 4. Inhibitory effect of 10 μM FMRFamide on the activity of swim motoneuron **R2A**, located in the pedal ganglion. Note the hyperpolarization of the motoneuron and disappearance of synaptic inputs. After a 5-min wash, impulse activity was restored. Scale bars = 20 mV, 3 s.

(Fig. 4). FMRFamide produced hyperpolarization of swim motoneurons. It also appeared to inhibit the swim pattern generator (SPG), as evidenced by the loss of the synaptic pattern of activity produced by the SPG in swim motoneurons.

FMRFamide immunolocalization in Clione

Central nervous system. Immunoreactive somata were found on dorsal and ventral surfaces of buccal, cerebral, pedal, and intestinal ganglia (Fig. 5).

Buccal ganglia. The buccal ganglia contained two pairs of immunoreactive cells on the ventral anterior surface and a few cells on the dorsal surface. The most conspicuous of the latter cells were a pair of large (80 μm in diameter) neurons found near the posterior margin (Fig. 5; 6C, D). In addition, an asymmetrical immunoreactive neuron was found on the medial margin of the right buccal ganglion (Fig. 5).

Cerebral ganglia. Several immunoreactive cells were found in the cerebral ganglia (Fig. 5). These included two clusters of large cells (60–80 μm) at the anterior margin of the ganglia, which showed stable, weak fluorescence in each preparation. A group of intermediate-size neurons (40–50 μm) was revealed in the middle of each ganglion on the dorsal surface. On the ventral surface, two notable immunoreactive cells with bright fluorescence were revealed in the central part of the ganglia, along with a pair of large cells (up to 100 μm), showing weak fluorescence, in the posteriolateral margins. Small somata included a tight cluster on the posteriomedial margin, near the cerebral commissure. Also, a large asymmetrical cell body, 70 μm in diameter, with bright fluorescence, was localized in the posteriomedial margin of the ganglia.

Pedal ganglia (Fig. 5). In the pedal ganglia, two cells showing bright fluorescence were found in the anterior region, and two additional cells were localized in the lateral region of the dorsal surface. Also, a tight cluster of small neurons was revealed in the anteriolateral part of the dorsal surface of each pedal ganglion. On the ventral surface, two labeled neurons were found in the anterior region, and a group of neurons was localized in the posteriomedial region. A cluster of small neurons was also revealed in the lateral region of the ganglia.

Intestinal ganglia (Fig. 5). Large (up to 100 μm in diameter) immunoreactive cell bodies with asymmetrical positions were localized on the dorsal and ventral surfaces of intestinal ganglia. Also, on the posterior margin of the intestinal ganglia, a pair of symmetrical large neurons showed stable, extremely bright fluorescence in each preparation. These cells sent thick axons into intestinal nerve N12, which innervated the esophagus (according to Wagner, 1885). In addition, two small cell bodies were found in the pleural ganglia. All ganglia contained a dense network of neuropilar immunoreactive processes.

Head. The head of *Clione* had a dense network of immunoreactive processes partly originating from immunoreactive fibers that run from cerebral ganglia to the head nerves and cerebro-buccal connectives and from buccal ganglia to the buccal mass. In addition, the buccal mass contained many extraganglionic immunoreactive somata (Fig. 6). Three groups of small immunoreactive cell bodies were found in the middle of the buccal mass, between the hook sacs (Fig. 6). The first group consisted of a pair of symmetrical neurons found on the ventral surface of the buccal mass far from the buccal ganglia. Two other groups included two symmetrical pairs of clusters found on the dorsal surface, closer to the buccal ganglia, and consisted of 3–5 neurons per cluster. Cells from each cluster sent axons to the ipsilateral buccal ganglion. Additional multiple cell bodies were found around the mouth and radula, most associated with the lips (Fig. 6A). A dense network of immunoreactive processes was revealed in the head skin (Fig. 7A) originating from intensely immunoreactive fibers that crossed the whole thickness of the head wall (Fig. 7B).

Buccal cones. Buccal cones had a dense network of immunoreactive fibers arranged in a longitudinal, compressed network (Fig. 7C, D). It is important to stress that these immunoreactive fibers were restricted to the oral side of each buccal cone—the side that makes contact with the prey. There are three groups of muscles in the

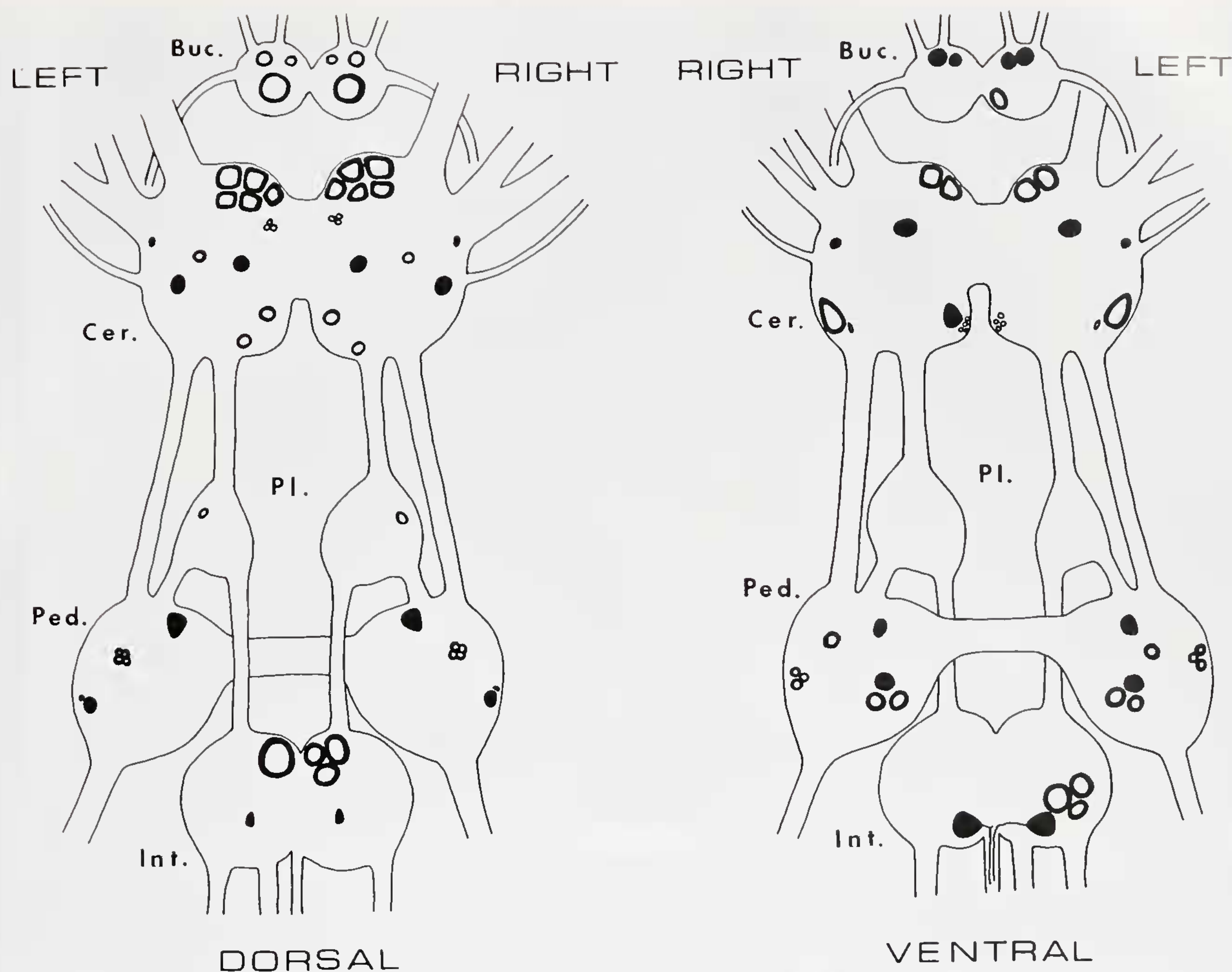

Figure 5. A map of FMRFamide immunoreactive cells on the dorsal and ventral surfaces of central ganglia including buccal (Buc), cerebral (Cer), pedal (Ped), pleural (Pl), and intestinal (Int) pairs. Cells filled in black showed a high level of immunoreactivity; white cells showed a lower level.

buccal cones as revealed by light and electron microscopy: circular smooth muscles and smooth and striated longitudinal muscles (Fig. 8A–C). Immunoreactive processes were primarily associated with bundles of striated longitudinal muscles. Electron microscopical examination of buccal cones revealed immunoreactive terminals adjacent to the striated longitudinal muscle cells (Fig. 8D).

Wings. The wings of *Clione* also contained networks of immunoreactive fibers originating from the pedal ganglia and wing nerves (Fig. 9A). Material sectioned for both light (Fig. 9B) and electron microscopy revealed that immunoreactive processes are associated only with retractor muscles of the wings: transverse and longitudinal smooth retractor muscles (classification according to Huang and Satterlie, 1989).

Effects of gamma-aminobutyric acid

Gamma-aminobutyric acid (GABA) depolarized and activated the A neurons but hyperpolarized and inhibited the **B** neurons (Fig. 10). To test the direct sensitivity of A and **B** neurons to GABA, they were chemically isolated with high Mg^{++} or 0.5 mM Cd^{++} solutions. In all cases, GABA depolarized A neurons and hyperpolarized **B** neurons (Fig. 10B). Local application of GABA onto the somata of recorded A and **B** neurons produced similar results (Fig. 10A). Both GABA effects, depolarization of A neurons and hyperpolarization of **B** neurons, were associated with increases in membrane conductance (Fig. 10C).

Both A and **B** neurons were very sensitive to GABA. The depolarizing effect of GABA on A neurons was noted with concentrations as low as 0.3 μM. Strong activation of spike activity in A neurons was typically produced with GABA concentrations of 1 μM and higher. Hyperpolarizing responses were noted in **B** neurons with GABA concentrations as low as 0.1 μM. GABA at a concentration of 1 μM had a powerful inhibitory effect on **B** neurons,

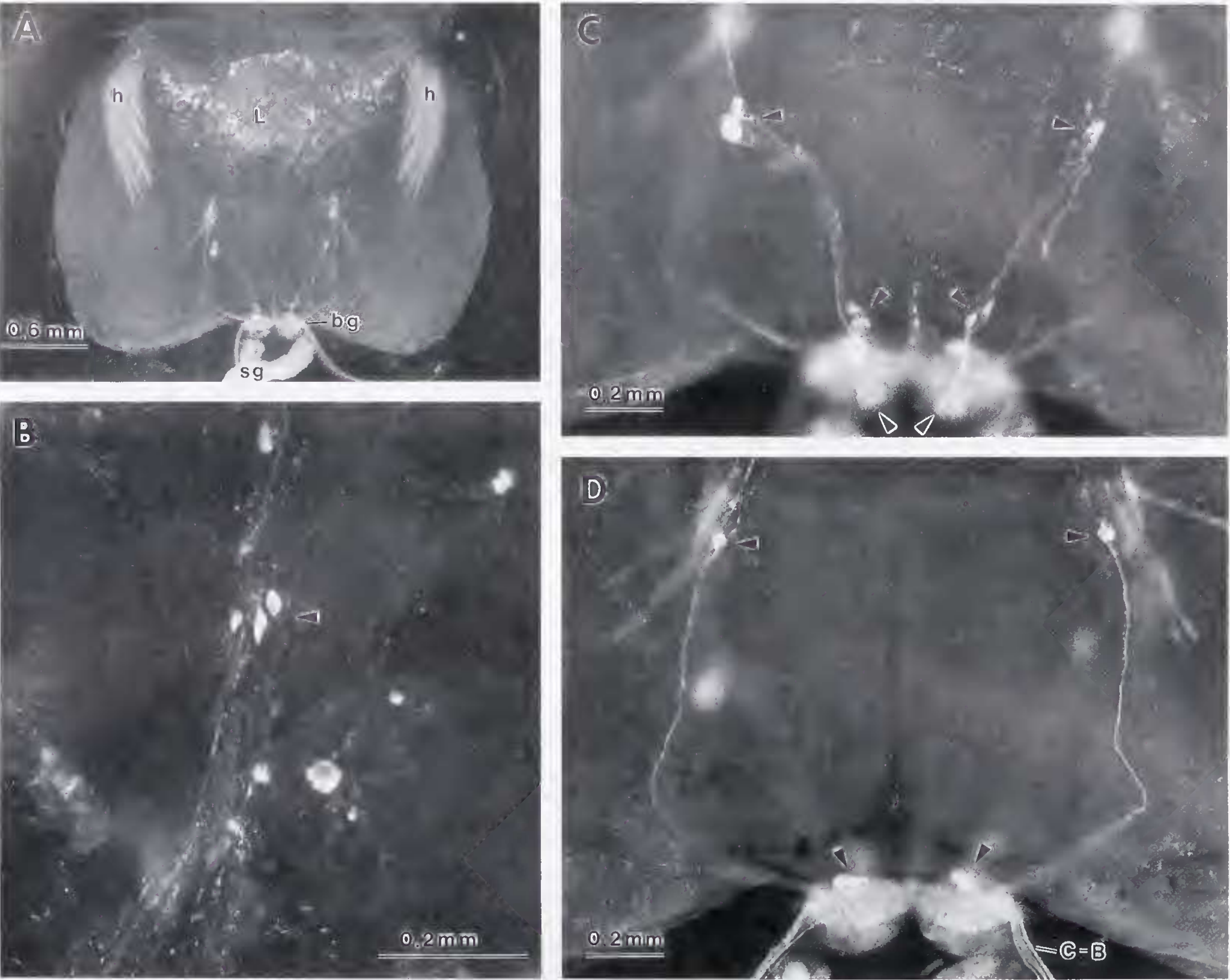

Figure 6. Photomicrographs of FMRFamide immunoreactivity in the buccal ganglia and buccal mass. (A) General view of the ventral surface: bg, buccal ganglia; sg, salivery gland; h, hooks; L, lips. (B) Higher magnification of the buccal mass surface showing extraganglionic immunoreactive cell bodies (arrow) and fibers. Dorsal (C) and ventral (D) surfaces of the buccal ganglia and close-by regions of buccal mass: arrows show immunoreactive cell bodies, C-B cerebro-buccal connective.

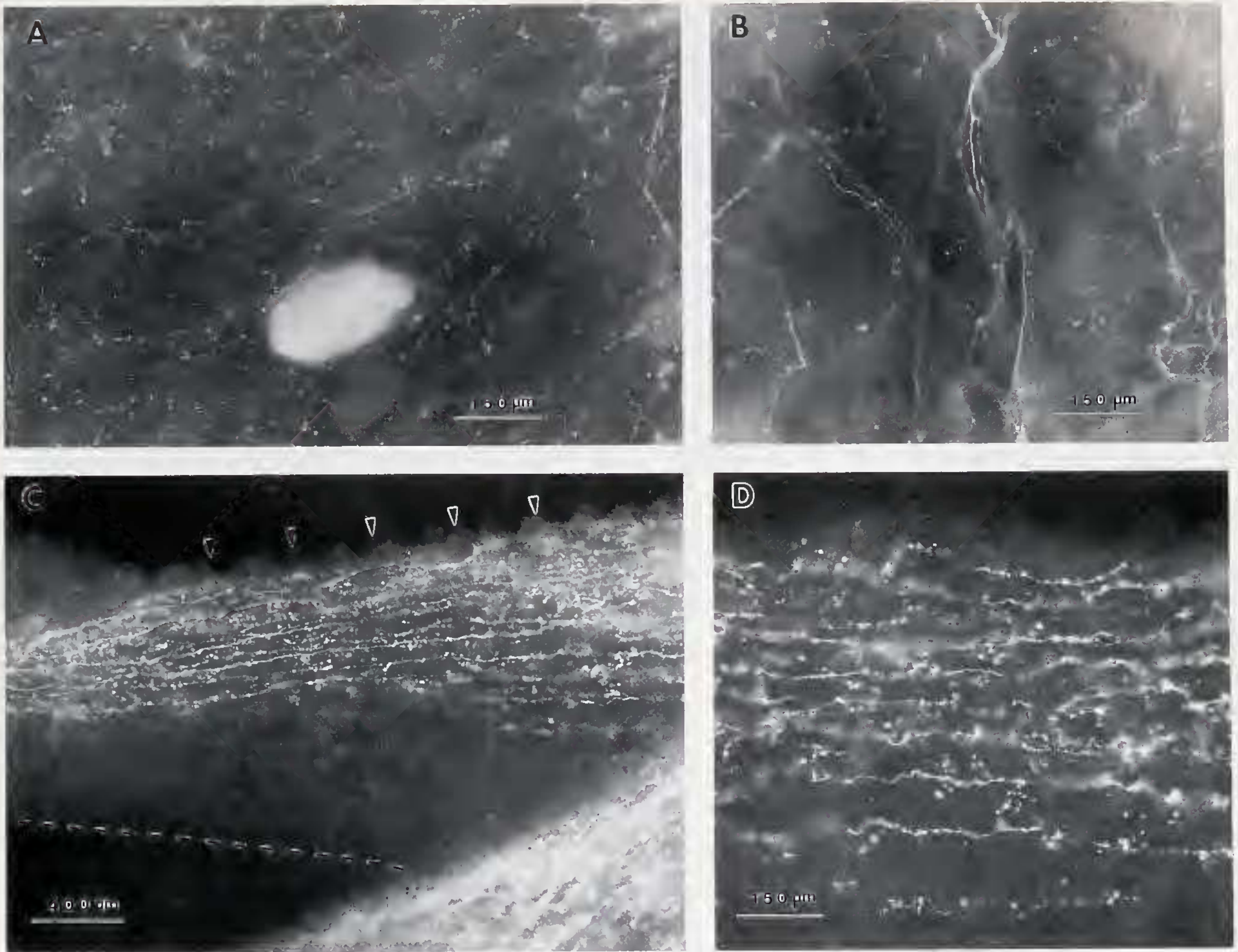

Figure 7. FMRFamide immunoreactivity in the head. (A) Network of immunoreactive fibers on the surface of the head skin. (B) Immunoreactive processes run through the entire thickness of the head wall. (C, D) Dense network of longitudinally oriented immunoreactive fibers in buccal cones. Note that fibers are localized only on the internal, oral side of the buccal cone (indicated by arrows). The extent of the external (aboral) side of the cone is indicated by the dashed line in C.

producing hyperpolarizing responses up to 10 mV in amplitude. During GABA application, **B** neurons received two types of inhibitory influence. First, the direct effect of GABA was membrane hyperpolarization. Second, activation of **A** neurons resulted in powerful synaptic inhibition of **B** cells (Fig. 10D).

Bath application of 1 μM (or higher) GABA in seawater produced strong activation of **A** neurons and inhibition of **B** neurons (Fig. 10D). GABA thus produced a reciprocal effect on **A** and **B** neurons. The overall behavioral effect of GABA perfusion was the opening of the oral skin folds and protraction of buccal cones. These effects persisted while GABA was present in the recording chamber.

Possible GABAergic nature of IPSPs in B neurons

GABA was the only transmitter studied that caused hyperpolarization of **B** neurons. Other neurotransmitters tested—including FMRFamide, serotonin, dopamine, acetylcholine, SCP_B, and L-glutamate—depolarized **B** neurons. Experiments were therefore conducted to test whether GABA is the transmitter released onto the **B** neurons during **A** neuron activity.

The reversal potentials for GABA-induced hyperpolarization (-60 mV $\pm$ 3 mV, n = 7) and **A**-neuron-induced IPSPs (-60 mV $\pm$ 2 mV, n = 10) appeared identical and indicated a selective increase in membrane permeability to chloride ions. To increase the intracellular concentration of chloride ions, the recording electrodes were filled with 3 M potassium chloride instead of 2 M potassium acetate. Leakage of chloride ions from the tip of such electrodes would be expected to cause an increase of intracellular chloride concentration and a shift in the reversal potential of a chloride-mediated response in the

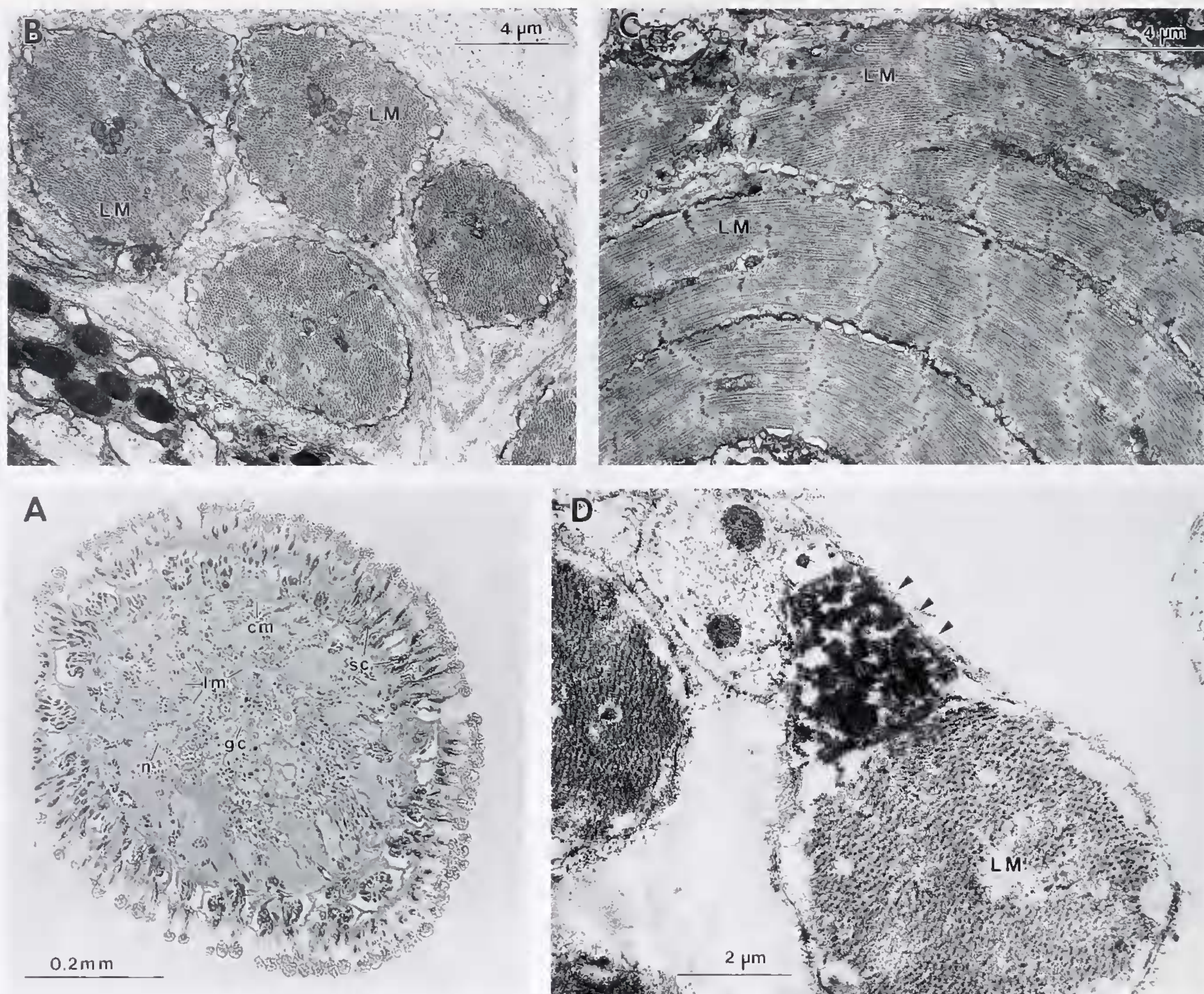

Figure 8. Buccal cones. (A) Light photomicrograph of a buccal cone cross section: lm, longitudinal muscles; cm, circular muscles; n, nerve branch; gc, gland cells; sc, secretory cells; the hemocoelic space is not seen since the buccal cone was in a retracted position when fixed. (B) Electron micrograph of a buccal cone cross section: LM, longitudinal muscle cells. (C) Electron micrograph of a longitudinal section of a buccal cone showing cross-striation of the longitudinal muscle cells. (D) Electron micrograph of a buccal cone cross section showing an immunoreactive terminal (arrows) adjacent to a striated longitudinal muscle cell (LM).

depolarizing direction. Results from one experiment using KCl electrodes placed in **B** neurons are shown in Figure 11A. Just after penetration of the cell, normal IPSPs were recorded following **A** neuron activation. After 10 min, similar stimulation did not produce a change in the **B** neuron membrane potential. Thirty minutes after penetration, **B** neuron IPSPs were reversed, and after 50 min the amplitude of the depolarizing responses was large enough to produce strong activation of the **B** neuron (Fig. 11A). Such experiments suggest that chloride ions are involved in generating the **B** neuron IPSPs.

Similar changes were observed in GABA responses of **B** neurons recorded with KCl electrodes (Fig. 11B). Just after penetration, GABA perfusion produced strong hyperpolarization of **B** neurons. After 10 min, the hyperpolarizing responses disappeared. Twenty to forty minutes after penetration, GABA application began to produce strong depolarization of **B** neurons (Fig. 11B). In several experiments, we tested both IPSPs and GABA responses in the same **B** neuron and observed simultaneous changes in both IPSPs and GABA responses. During these experiments, the baseline membrane potential did not change

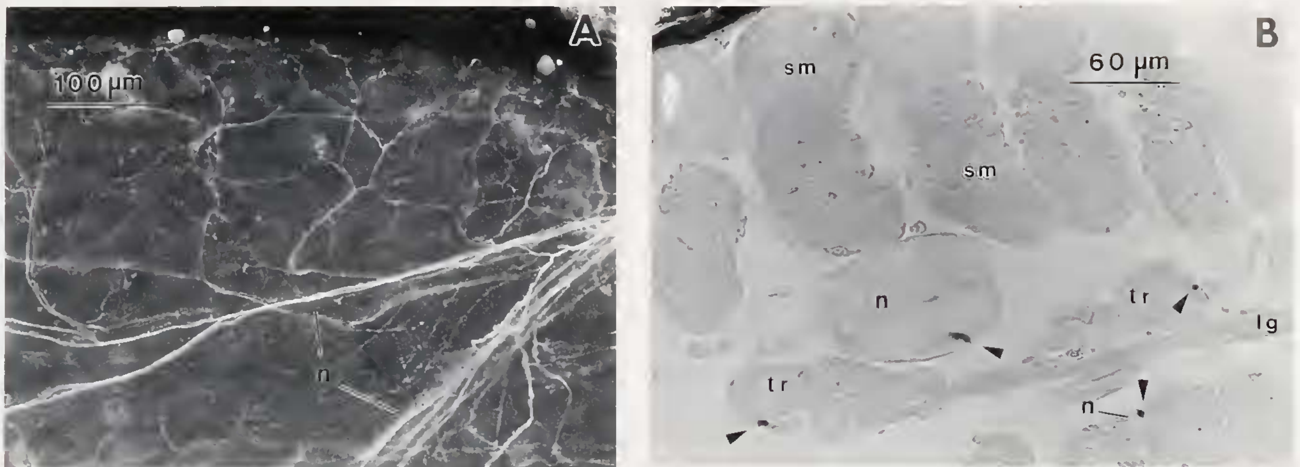

Figure 9. FMRFamide immunoreactivity of the wings. (A) Immunoreactive fibers (fluorescent secondary antibody) enter the wing through the pedal wing nerve and branch extensively inside the wing: n, two main branches of the wing nerve within the left wing. The optical section is parallel to the dorsal surface of the wing. (B) Transverse section of the wing: arrows show immunoreactive fibers (peroxidase-labeled secondary antibody); sm, swim muscles; tr, transverse retractor muscles; lg, longitudinal retractor muscles; n, nerve.

by more than 4 mV. These data suggest that the **B** neuron IPSPs and GABA responses involve the same ionic mechanism: an increase in chloride permeability.

The final test involved use of different GABA antagonists, specifically including blockers of GABA-activated chloride channels, such as picrotoxin. Picrotoxin did not block **B** neuron IPSPs, even at concentrations of 1 m*M*. Similarly, bicuculline (a specific blocker of $GABA_A$ receptors) at concentrations up to 1 m*M* did not influence the IPSPs. Because these results seemed to contradict the initial suggestion of a GABAergic nature of IPSPs in **B** neurons, the direct GABA responses of **B** neurons were examined. Picrotoxin did not alter the GABA-induced responses (GABA concentration of 10 μ*M*), even in concentrations of 1 m*M*. GABA-induced hyperpolarizations were also not influenced by pentylenetetrazole, another blocker of GABA-activated chloride channels, even when the concentration was 10 times higher than the GABA concentration. GABA responses were also unaltered by bicuculline at 1 m*M*, when GABA concentration was 10 μ*M*. These results were repeated in over 35 preparations during three experimental seasons. GABA receptors on **B** neurons thus are not sensitive to the GABA antagonists picrotoxin, pentylenetetrozole, or bicuculline. The data showing that picrotoxin did not block either chloride-dependent GABA responses or IPSPs in **B** neurons strengthen the initial suggestion of the GABAergic nature of **B** neuron IPSPs. However, the question of whether GABA participates in producing IPSPs in **B** neurons cannot be resolved by pharmacological methods. Only identification of particular GABAergic neurons that receive excitatory inputs from **A** neurons and monosynaptically inhibit **B** neurons can give a final answer.

Discussion

FMRFamide

FMRFamide has potent effects on central neurons of many mollusks. For example, some neurons of *Helix* are excited by FMRFamide, others are inhibited, and some show biphasic responses (Cottrell, 1983; Cottrell *et al.*, 1984; Walker *et al.*, 1981). In *Clione*, FMRFamide depolarized **B** neurons and strongly hyperpolarized **A** neurons. The reversal potential for FMRFamide responses in **A** neurons was about −80 mV, a value that is similar to the theoretical equilibrium potential for K^+ ions in many cells. This would suggest that the FMRFamide responses in *Clione* **A** neurons are potassium mediated, as in other molluscan neurons (Cottrell *et al.*, 1984; Boyd and Walker, 1985; Belardetti *et al.*, 1987; Brussard *et al.*, 1988). Further evidence for a potassium dependency to this response might be that the FMRFamide-induced hyperpolarizations were blocked by TEA and 4-AP, agents that are known to block voltage-activated K^+ currents (Hille, 1992). However, because those agents have not been reported to block ligand or second-messenger-activated K^+ currents, their action here may represent either a novel action on ligand-activated K^+ currents or an indirect effect. We cannot, as yet, distinguish between these possibilities. FMRFamide was the only active agent tested that caused a hyperpolarization in **A** neurons. Two types of naturally occurring IPSPs, fast and slow, were described in **A** neurons, although their source is not known. It is possible that some of the inhibitory inputs are due to activity in FMRFamidergic neurons. Many neurons throughout the central nervous system of *Clione* have been shown to have FMRFamide immunoreactivity, including

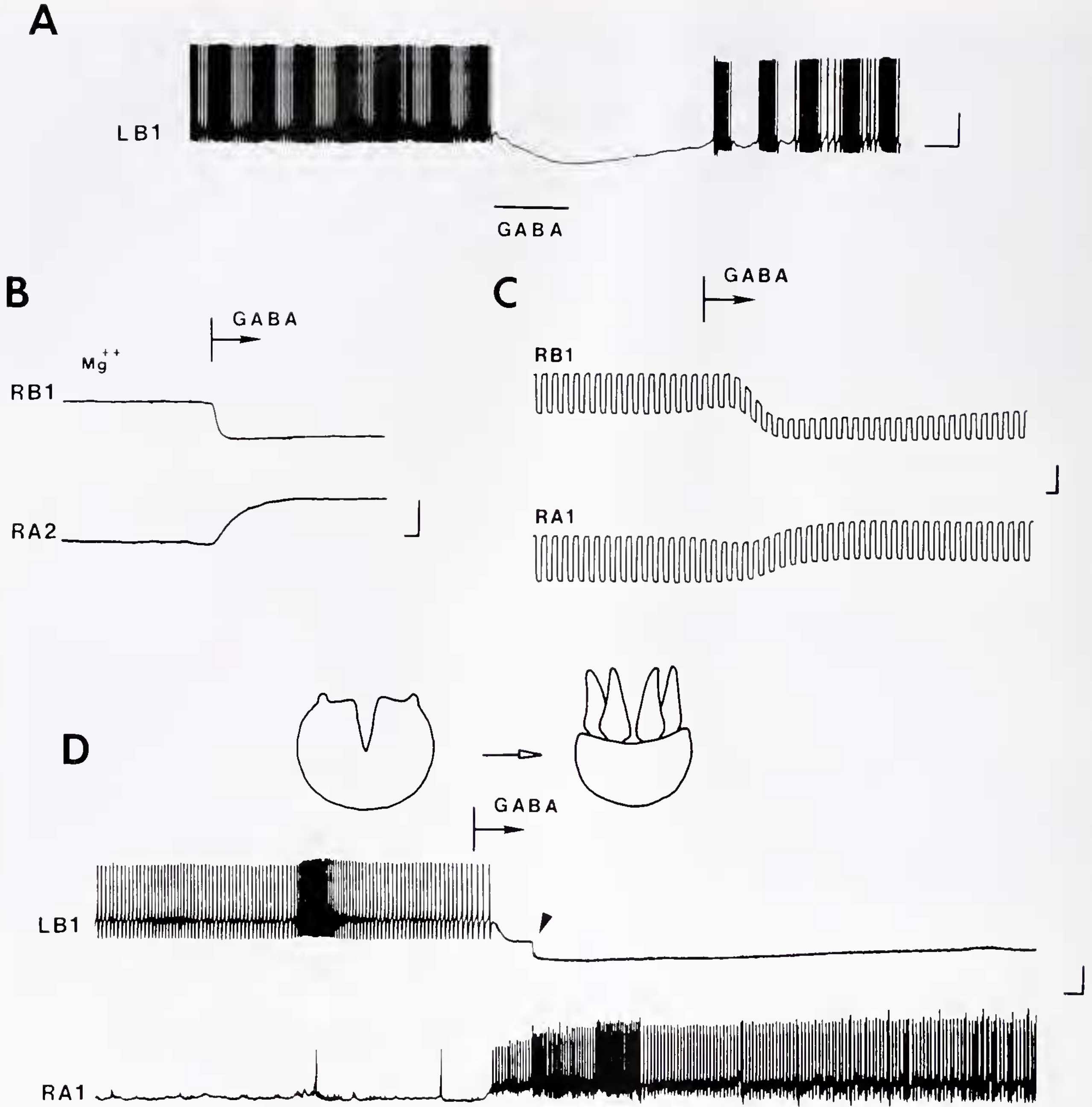

Figure 10. Effects of GABA on A and B neurons. (A) Local application of GABA from a glass microelectrode onto the soma of neuron **LB1**. The duration of the GABA pulse is indicated by the bar. Scale bars = 20 mV, 2 s. (B) Effect of 5 μM GABA on neurons **RA2** and **RB1** in high Mg^{++} solution. Note that GABA depolarizes **A** neurons and hyperpolarizes **B** neurons. Scale bars = 10 mV, 1 s. (C) GABA-induced hyperpolarization of B neurons and depolarization of **A** neurons are associated with the increases in membrane conductance. Two intracellular electrodes were used in these experiments; one for recording membrane potential (shown in figure), and a second for injecting negative current pulses. Current pulses: neuron **RB1**, −1 nA; neuron **RA1**, −2 nA. Scale bars = 10 mV, 2 s. (D) Effect of 2 μM GABA on neurons **LB1** and **RA1** in seawater. Note the initial smooth hyperpolarization and the delayed appearance of inhibitory postsynaptic potentials (shown by arrow) in **B1** neuron initiated by **A** neurons. The drawings above the record indicate observed activity of the head before and after GABA application. Before application skin folds were closed and buccal cones were withdrawn; after application skin folds were open and buccal cones were partially extruded. Scale bars = 20 mV, 2 s.

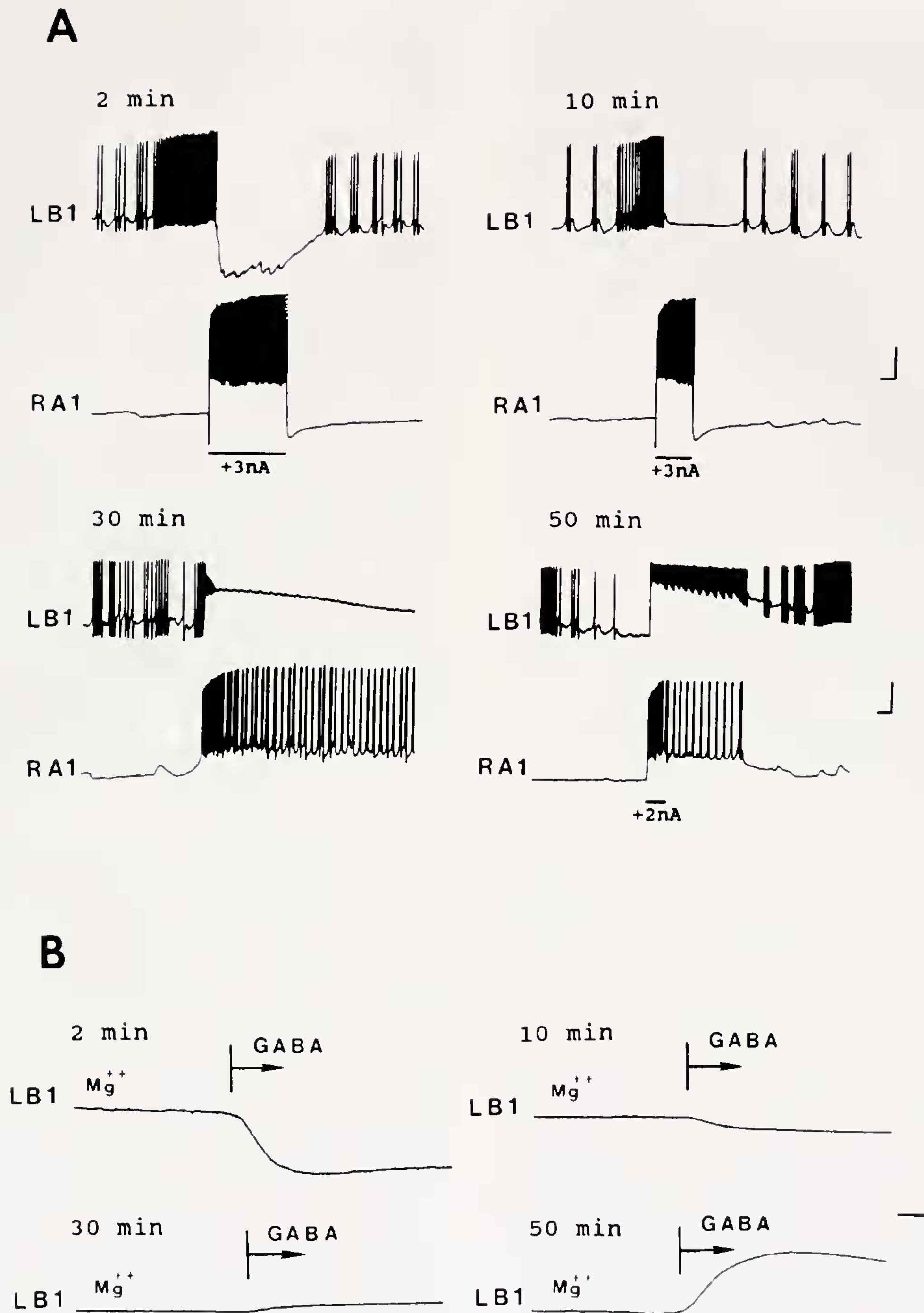

Figure 11. Recordings from neuron **LB1** with microelectrodes filled with 3 *M* KCl. (A) Two minutes after penetrating the **B** neuron, a burst of action potentials in **RA1** produced a hyperpolarizing response; 10 min after penetration, the hyperpolarizing response was eliminated; 30 min after penetration, induced spike activity in **RA1** produced a depolarizing response in **LB1**, 50 min after penetration, the depolarizing **B** neuron response was sufficient to trigger spike activity in **LB1**. Scale bars = 10 mV, 2 s. (B) Direct GABA responses in **LB1** following application of 5 μM GABA. The recordings were made in high Mg^{++} seawater. The beginning of GABA application is indicated by the vertical bar with the attached arrow. Two minutes after penetration with the KCl electrode, a hyperpolarizing response was induced. This response was reduced at 10 min and reversed at 30 min. Fifty minutes after penetration, GABA perfusion produced a large depolarizing response. Scale bars = 10 mV, 2 s.

cells of the buccal, cerebral, pedal, and intestinal ganglia. The map of FMRFamide immunoreactive neurons can now be used to search for candidate neurons that influence **A** and **B** neuron activities.

The effect of FMRFamide on **A** and **B** neurons is well coordinated to produce inhibition of buccal cone extrusion (hyperpolarization of **A** neurons) and enhancement of buccal cone retraction (depolarization of **B** neurons). The overall behavioral response, withdrawal reaction of the buccal cones, was always observed during FMRFamide perfusion. Similar inhibitory effects of FMRFamide on the components of feeding behavior have been described for several other molluscs. Micromolar concentrations of FMRFamide inhibited patterned motor activity, which underlies feeding behavior, in *Helisoma* buccal ganglia and suppressed activity in salivary gland cells (Murphy *et al.*, 1985; Bulloch *et al.*, 1988). The inhibitory influence of FMRFamide on the feeding motor program was demonstrated in lip-brain preparations of *Limax* (Cooke *et al.*, 1985; Cooke and Gelperin, 1985). FMRFamide also inhibited spontaneous and induced contractions of the anterior gizzard of *Aplysia* (Austin *et al.*, 1983).

Immunolocalization of FMRFamide in cells in buccal ganglia, as well as in extraganglionic neurons in the buccal mass and mouth region, and the dense network of immunoreactive fibers in the buccal muscles suggest a physiological role for this neuropeptide in the regulation of feeding behavior of *Clione*. Similarly, FMRFamide immunoreactivity has been observed in a number of neurons in buccal ganglia and in buccal muscles of *Aplysia* (Lloyd *et al.*, 1987). Of particular interest is localization of intensely immunoreactive fibers in *Clione* buccal cones. FMRFamide immunoreactive terminals were found to innervate cross-striated longitudinal muscle cells, which underlie movements of protracted buccal cones. Contraction of the longitudinal muscles on the oral side of buccal cones would produce catching and holding movements of the cones, which is observed during the acquisition and manipulation phases of feeding behavior. FMRFamide immunoreactive fibers were found only on the oral side of each buccal cone. This localization of immunoreactive processes suggests that FMRFamide participates in control or modulation of buccal cone movements during feeding behavior of *Clione*.

Bath application of FMRFamide also led to the inhibition of swimming, termination of which is a component of withdrawal behavior in *Clione*. FMRFamide immunoreactive nerve fibers, observed in the wings of *Clione*, were associated with retraction musculature. Excitatory effects of FMRFamide on retractor muscles have been demonstrated on the anterior byssus retractor muscle of *Mytilus edulis* (Painter, 1982) and on *Helix* tentacle retractor muscle (Cottrell *et al.*, 1983). FMRFamide therefore may act as a mediator involved in inhibitory control of different components of feeding behavior in *Clione*.

Gamma-aminobutyric acid

GABA has been found to excite some gastropod neurons and to inhibit others (Walker *et al.*, 1975; Yarowsky and Carpenter, 1978; Oomura *et al.*, 1979). Yarowsky and Carpenter (1978) identified five types of responses in *Aplysia* neurons, the most common of which was a fast chloride-mediated hyperpolarization with a reversal potential of −58 mV. Picrotoxin (1–100 μM) and bicuculline (10–100 μM) blocked the chloride-mediated inhibition produced by GABA.

In *Clione*, GABA depolarized and activated **A** neurons and hyperpolarized **B** neurons. The hyperpolarizing response of **B** neurons had a reversal potential of −60 mV and was chloride-mediated. Likewise, IPSPs in **B** neurons, induced by **A** neuron activity, had reversal potentials of −60 mV, and were linked with activation of chloride channels. These data, plus the observation that GABA was the only transmitter tested that inhibited **B** neurons, suggest that GABA might be the transmitter released during the **A**-to-**B** neuron inhibition. GABAergic neurons have been localized by immunohistochemical methods in *Clione* in the cerebral, pedal, and buccal ganglia (Arshavsky *et al.*, 1993).

However, picrotoxin, a blocker of GABA-activated chloride channels (Nistri and Constanti, 1979; Simmonds, 1983; Tallman and Gallagher, 1985), did not block either **B** neuron IPSPs or the direct inhibitory action of applied GABA, even in concentrations of 1 mM. Bicuculline, a specific antagonist of inhibitory $GABA_A$ receptors, also did not block the IPSPs or GABA responses. The fact that bicuculline, picrotoxin, and pentylenetetrazole (another tested GABA chloride-channel blocker) did not have any effect on chloride-mediated GABA inhibition in **B** neurons was unusual, but not unique. GABA-activated chloride-conductance increases that are insensitive to picrotoxin have been described in the stomatogastric ganglion of the crab *Cancer pagurus* (Marder and Paupardin-Tritsch, 1978) and in neuromuscular preparations of the spiny lobster, *Panulirus* (Albert *et al.*, 1986). It was also reported that bicuculline had no effect on GABA-mediated IPSPs in the locust (Watson and Burrows, 1987) and did not inhibit the binding of 3H-GABA to a putative GABA receptor in cockroach nerve cord extracts (Lummis and Sattelle, 1985). The **B** neurons of *Clione* may represent another model for studying such unusual GABA inhibitory responses.

One of the most important aspects of the GABA effect in *Clione* is the coordinated influence on **A** and **B** neurons. GABA depolarized and activated **A** neurons but hyperpolarized **B** neurons, which also received powerful IPSPs

from the activated A cells. The overall effect is to turn off the system that is responsible for buccal cone withdrawal while activating the system that triggers food acquisition. During GABA perfusion, it was always possible to observe opening of the oral skin folds and protraction of the buccal cones.

GABA is usually considered a classical local neurotransmitter, and only a few studies have shown it to have widespread behavioral effects. GABA was shown to have an excitatory effect on patterned feeding motor activity in *Helisoma trivolvis;* GABA activated the central pattern generator in buccal ganglia in quiescent preparations and increased the rate of patterned activity in activated preparations (Richmond *et al.*, 1986). In *Limax*, however, GABA was reported to suppress the feeding motor program in a lip-brain preparation (Cooke *et al.*, 1985). In *Clione*, GABA injection into intact animals strongly activated feeding behavior, including protraction of buccal cones (Arshavsky *et al.*, 1993). Arshavsky *et al.* (1993) also described the excitatory effect of GABA on locomotion, on the feeding rhythm generator of the buccal ganglia, and on cerebral neurons, which appear to be some of the A neurons identified in our previous work (Norekian and Satterlie, 1993).

GABA and FMRFamide thus evoke functionally opposite behavioral effects in *Clione* through coordinated changes in the activities of three groups of neurons. FMRFamide inhibits prey-capture reactions and initiates withdrawal reactions: retraction of buccal cones through hyperpolarization of A neurons and depolarization of B neurons, and inhibition of swimming. GABA, on the contrary, initiates feeding reactions through powerful extrusion of the buccal cones due to strong activation of A neurons and direct inhibition of B neurons.

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